

Production of Milk Clotting Enzyme by *Penicillium camemberti* using Whey medium

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Abstract: Milk clotting enzyme is commercially known as Rennet which is composed of Rennin and Pepsin. It plays a vital role in Cheese making with good flavor and fine texture. The use of cheaper substrates instead of synthetic medium such as glucose, sucrose, etc will result lower cost of the final product. Approximately half of the cheese whey produced worldwide is discarded without treatment. The utilization of the whey to valuable bio products is the best way to avoid the dairy waste pollution. *Penicillium camemberti*, a fungal culture was used for the production of milk clotting enzyme by using whey as a substrate in this study. *Penicillium camemberti* has the ability to produce a high milk clotting enzyme in submerged fermentation.

Keywords: Milk clotting enzyme, Rennin, Proteolytic activity. Response surface methodology.

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I. Introduction

The development of food technology leads to increase the applications of food grade enzymes. Enzymes for food applications are obtained from animal, plant and microbial sources and the hydrolases play vital role in the food industries. Milk clotting enzyme is biochemically known as Aspartic protease, an extracellular hydrolase enzyme. Bovine Chymosin in the form of Calf Renet has been used for cheese making, dating back to approximately by 6000 BC (Sirmayegin, *et al.*, 2011) Traditional cheese production by using Bovine rennet is the oldest method. Natural calf rennet is extracted from the fourth stomach (abomasum) of milk fed young calves. The limited availability of animal rennet leads to a search for alternate rennet substitutes for cheese making industry.

Many plants have coagulating properties for example, extract of fig juice, Papaya & Pine apple to coagulate milk. Microbial rennet are produced from the microorganisms both fungi and bacteria. Some molds such as *Rhizomucormiehei* are able to produce milk clotting enzyme. Micro organisms, *Endothia Parasitica*, *Bacillus reuses*, *mucorpusilus*, *Cryptococcus albidus* and *Mucormehi* are known to produce milk clotting enzymes which substitute calf rennet (Fox, 1991; and Bailey *et al.*, 1988). Most of the plant rennets have proved not suitable for cheese making because of the bitter taste. But the microbial rennets are more promising because its cheaper production, greater biochemical diversity and the genetic modification is easier (Tanboly *et al.*, 2013)

Whey is the by-product of cheese and curd manufacturing, once it was considered as a waste product. The discovery of whey as a functional food with nutritional applications elevated whey to a co-product in the manufacturing of cheese (Walzem *et al.*, 2002). Milk contains two sources of protein, the caseins and whey. During processing, the caseins are the proteins responsible for making curds, while whey remains in an aqueous environment. Whey is a major source of lactose, good source of valuable protein and minerals and water soluble vitamins (Bande, 2011). The biological components of whey, including lactoferrin, beta lactoglobulin, alpha-lactalbumin, glycomacropptide, and immunoglobulins, have immune-enhancing properties. In addition, whey act as an antioxidant, antihypertensive, antitumor, hypolipidemic, antiviral, antibacterial, and chelating agent. A number of clinical trials have successfully been performed using whey in the treatment of cancer, HIV, hepatitis B, cardiovascular disease, osteoporosis, and as an antimicrobial agent. (Keri Marshall, ND, MS., 2004).

Dairy industries dispose strong waste water characterized by high BOD and COD representing their high organic content (Demirel *et al.*, 2005). Worldwide production of whey is about 145 million tons, with 60% recovered by several methods and 40% discarded directly into rivers (Celia Maria *et al.*, 2001.) The utilization of whey for the production of valuable commercial products is the best solution for the environmental problem caused by the disposal of whey (Morr *et al.*, 1993).

In the present study, different medium namely basal medium, casein and lactose along with basal medium in whey medium and plain whey were studied under stationary and shaking conditions for the production of milk clotting enzyme by *Penicillium camemberti*. The aim of this study was to find out the optimum process conditions for the selected operating variables namely initial substrate concentration, initial

pH, temperature and biomass concentration for the maximum production of milk clotting enzyme using whey as substrate by Response surface methodology.

Response surface methodology (RSM) is an empirical statistical method utilized for multiple regression analysis of quantitative data obtained from statistically designed experiments by solving the multivariate equations simultaneously. The response surfaces are the graphical representation of the quadratic equations to explain the individual and cumulative effect of the test variable response surfaces and to find out the interaction between the test variables. (Khuri. 1982., Montgomery.1932) .

II. Materials and methods

2.1 Microorganism.

The fungal culture *Penicillium camemberti* (MTCC 418) was obtained from MTCC Chandigarh, India. This culture was maintained by sub culturing periodically at 30°C for 5 days and stored at 4°C.

2.2 Growth and Production medium

The composition of the growth and fermentation medium were mentioned below (g/l) Czapek concentrate 10 ml; K₂HPO₄ 1.0; Yeast extract 5.0; Sucrose 30.0; Distilled water 1000ml. Composition of czapek concentrate (g/100ml) are NaNO₃ 30.0; KCl 5.0; MgSO₄·7H₂O 5.0; FeSO₄·7H₂O 0.1; The 250 ml Erlenmeyer Flask Contained 100ml of the medium and autoclaved for 15 min at 121k_{gf} which were then inoculated and incubated at 30°C for 5 days.

The production was carried out by using known volume of 5 days inoculums in the above medium using whey at 30°C both static and shaker at 120 rpm for 5 days. Fermentation medium of above composition namely plain basal medium, Lactose and Casein along with the basal medium in whey medium and plain whey were used for this study. All experiments were carried out in duplicate and repeated at least twice. Samples were taken from the solution at regular time intervals for the analysis of milk clotting activity, proteolytic activity, biomass concentration and protein content.

2.3 Experimental Design and Statistical Analysis

The factors affecting the production of milk clotting enzyme from whey by *Penicillium camemberti* was studied using Central Composite Design (CCD) experiments. The initial substrate concentration (A) g/l, initial pH (B), temperature (C) °C and biomass concentration (D) g/l were chosen as the independent variables as shown in Table 1. Milk clotting activity (Y) was chosen as the dependent output variable. An orthogonal 2⁴ full factorial central composite design with eight star points (α=2) and seven replication at the centre point, all in duplicates, resulting in a total of 31 experiments were used to optimize the chosen key variables for the production of Milk clotting enzyme in a batch reactor.

The experiments with various initial substrate concentrations (whey medium) namely 10, 20, 30, 40 and 50 (% v/v), different initial pH values of 5.0, 5.5, 6.0, 6.5 and 7.0, different temperatures of 30, 35, 40, 45 and 50°C and five different biomass concentrations of 3.0, 6.0, 9.0, 12.0 and 15.0 g/l were employed and varied simultaneously to cover the combinations of variables in the design. The range and the levels of the experimental variables investigated in this study were given in Table 1. The chosen independent variables used in this experiment were coded according to Eq. (1):

$$x_i = \frac{X_i - X_o}{\Delta x} \quad \dots (1)$$

Where x_i is the coded value of the i^{th} variable, X_i is the uncoded value of the i^{th} test variable and X_o is the uncoded value of the i^{th} test variable at the centre point

The behaviour of the system is explained by the following second- degree polynomial Eq. (2):

$$Y = \beta_o + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^k \beta_{ij} X_i X_j \quad \dots (2)$$

Where Y is the predicted response, β_o is the offset term, β_i is the coefficient of linear effect, β_{ii} is the coefficient of squared effect and β_{ij} is the coefficient of interaction effect. This regression model can be used to estimate the elliptical contours of a constant surface.

A statistical design package, Minitab 16 was used for regression analysis of the data obtained and to estimate the coefficients of the second-degree polynomial equation. The equations were validated by the statistical tests called the analysis of variance (ANOVA), to determine the significance of each term in the equation and to estimate the goodness of fit in each variable. Response surfaces were drawn to determine the individual and interactive effects of test variables on milk clotting activity.

2.4 Preparation of whey

The fresh milk whey was provided by **Ponlait Dairy products Ltd., Pondicherry, India**. To remove the suspended particles contained in raw whey, filtration step was performed by Whatmann No. 1 filter paper. The clarified whey was used as a substrate for milk clotting enzyme production.

2.5 Preparation of the Crude enzyme

The fermented medium was filtered to separate the biomass from the culture filtrate using whatman no 40 filter paper. The filtrate was centrifuged at 4°C for 10 min at 10000 rpm in the cooling centrifuge. Then the supernatant was used for the enzyme assays.

2.6 Analysis of crude enzyme

2.6.1 Estimation of Milk clotting activity:

Milk clotting activity (MCA) was determined by the method explained by Arima., etal(1964) and Balls.,etal(1937) using 0.1 (w/v) of rennin std and the substrate is 10g of skimmed milk powder in 0.01 mol. calcium chloride . The reaction mixture contains 5 ml of skim milk and 1ml of enzyme. It was kept at 37°C for MCA. The curd formation was observed by manually rotating the test tube from time to time. The end point is the semi liquefied film appears on the side of the test tube above the milk. The clotting time was noted.

$$MCU / mg = \frac{M}{T(\text{minutes}) \times W(g)} \quad (3)$$

Where M is the milk factor, T is the clotting time of sample (min) and Wis the grams of enzyme added to the substrate in 2.0 ml aliquot (g wt. x 2)

2.6.2 Estimation of Proteolytic activity

Proteolytic activity was determined by the Universal Protease activity assay by using casein as a substrate. The reaction mixture containing 5 ml of 0.65% pre incubated casein solution (37 °C/10min) and 1ml of enzyme was incubated for 10 min at 37°C. And 5 ml of TCA was added to stop the reaction and incubated at 37°C for 30 min. During the tyrosine standard was set up (0.2mg/ml) in the range of 0.1-0.5ml, made up to 2ml with distilled water. Then the test solutions are centrifuged at 4°C at 10000rpm for 10 min and the 2ml of aliquots are used for PA. To all the tubes (including standard), 5 ml of sodium carbonate, 1ml of Folin’s phenol is added and incubated at 37°C for 30 min . Then the optical density was measured at 660 nm by using uv-Biospectrophotometer.(Anson,1937.,Chwen-jenshieh etal.2009)

$$Units / ml \ enzyme = \frac{(\mu\text{mole tyrosine equivalents released}) \times (11)}{(1) \times (10) \times (2)} \quad (4)$$

Where 11 is the total volume of assay(ml), 10 is the time of assay as per the unit definition (min), 1is the volume of enzyme used(ml) and 2is the volume used in colorimetric detrmation(ml).

2.6.3 Determination of Protein

Protein was estimated by Lowry method (1951) by using BSA as a standard. The optical density was measured for 660 nm.

2.6.4 Estimation of Biomass concentration

Samples from the production medium were filtered through whatmann no .40 filter paper to separate the biomass. The settled biomass was collected and dried and expressing the dry weight as grams per litre of growth medium.

III. Results and Discussion

3.1 Effect of different medium components on the production of milk clotting enzyme

The effect of addition of lactose and casein along with the basal medium of whey, plain basal medium and plain whey medium under agitated and stationary condition for the production of milk clotting enzyme was evaluated with remarkable observation. Fig 1 shows the milk clotting activity and proteolytic activity levels obtained in a whey. The plain whey medium ,plain basal medium,casein and lactose along with the basal medium were denoted as M1, M2,M3 and M4 respectively. Maximum milk clotting enzyme concentration 0.50 units/mg was obtained for *P. camemberti* under stationary condition. Fig 2 clearly indicates the addition of casein gives the higher milk clotting activity than the lactose, plain basal medium and plain whey. Casein with other substrates is an effective enhancer of milk coagulation (silva et al.,2014). Fig 2 shows the biomass concentration under static and shaking conditions. Maximum biomass concentration of 35.8 g/l was obtained in the presence of casein, followed by 30.5 g/l in lactose , 28.5g/l in basal medium ,while plain whey had the least yield of 13.2 g/l under static conditions.

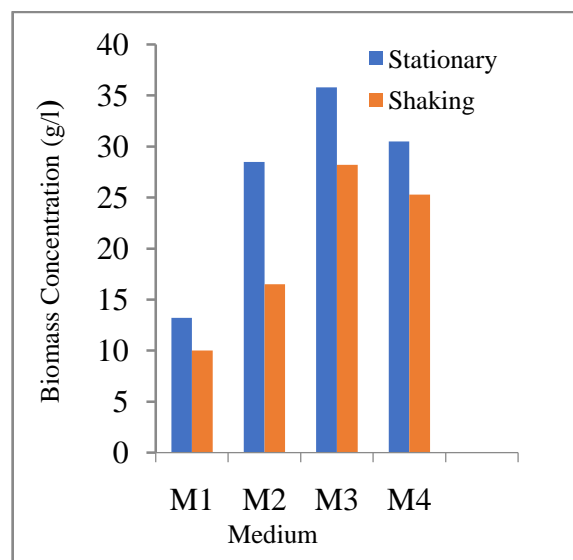
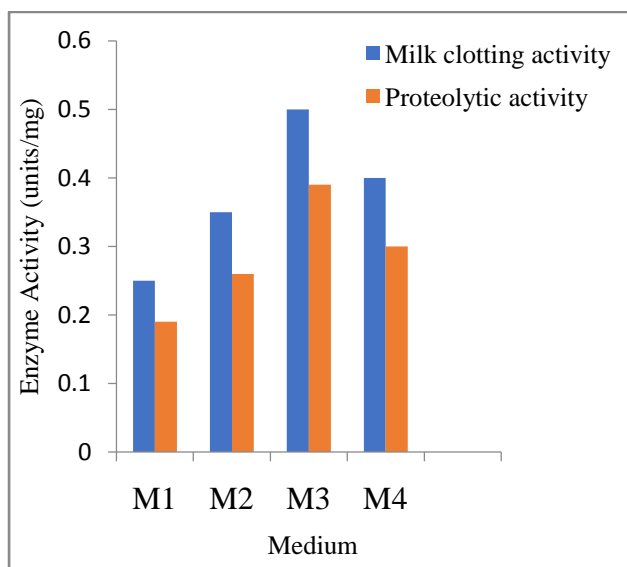


Fig 1 Effects of different medium components on milk clotting enzyme production by *Penicillium camemberti*

Fig 2 Effect of different medium components on biomass concentration by *Penicillium camemberti*

3.2 Central composite design and optimization using response surface methodology for the production of milk clotting enzyme

The coded values of the independent variables along with observed responses in each case were given in Table 2. By applying multiple regression analysis, a predictive quadratic model was fitted with experimental results, and the equation for the production of milk clotting enzyme was in the form of the following equation:

$$Y = 0.580 + 0.032A + 0.017B - 0.020C + 0.024D - 0.035A^2 - 0.016B^2 - 0.067C^2 - 0.019D^2 - 0.003AB + 0.010AC - 0.011AD + 0.016BC - 0.030BD + 0.036CD \dots (5)$$

where Y is the milk clotting activity (units/mg), A is the initial substrate concentration (whey medium) (%v/v), B is the initial pH, C is the temperature (°C) and D is the biomass concentration (g/l). The milk clotting observation during enzyme analysis for all 31 experimental run were given in Table 3. It was found that the milk clotting activity mainly depends on clotting time and enzyme concentration. Good coagulation was observed after 15 min when the pH was maintained at 6.0 and milk clotting was also observed at high temperature level within 20 min. Good clotting with fine curd within 15 min. was observed at the pH range of 6.0 at 40°.

Table 1 Central composite design for the production of milk clotting enzyme by *Penicillium camemberti*.

Independent Variable	Range and Level				
	-2	-1	0	+1	+2
Initial Substrate Concentration (whey medium) (%v/v) (A)	10	20	30	40	50
Initial pH (B)	5.0	5.5	6.0	6.5	7.0
Temperature (°C) (C)	30	35	40	45	50
Biomass Concentration (g/l) (D)	3.0	6.0	9.0	12.0	15.0

Table 2 Full factorial central composite design matrix of orthogonal values along with observed responses for the production of milk clotting enzyme

Run order	Independent Variable				Milk Clotting Activity (units/mg)	
	Orthogonal Value				Experimental	Predicted
	A	B	C	D		
1	-1	1	-1	1	0.480	0.412
2	1	-1	-1	1	0.480	0.492
3	0	0	0	-2	0.480	0.452
4	1	-1	1	-1	0.300	0.350
5	0	0	0	2	0.480	0.551
6	-1	-1	-1	-1	0.400	0.404
7	0	-2	0	0	0.436	0.471
8	-1	-1	1	1	0.522	0.442
9	2	0	0	0	0.533	0.502
10	0	0	0	0	0.582	0.581

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11	0	0	0	0	0.581	0.581
12	1	1	-1	-1	0.470	0.533
13	0	0	0	0	0.581	0.581
14	0	2	0	0	0.533	0.541
15	1	1	-1	1	0.436	0.426
16	0	0	0	0	0.400	0.371
17	-1	1	1	-1	0.400	0.371
18	1	-1	-1	-1	0.522	0.477
19	-1	1	1	1	0.436	0.456
20	0	0	0	0	0.580	0.581
21	1	1	1	-1	0.480	0.471
22	0	0	-2	0	0.342	0.354
23	1	1	1	1	0.533	0.572
24	-2	0	0	0	0.300	0.374
25	0	0	2	0	0.240	0.271
26	0	0	0	0	0.580	0.581
27	-1	1	-1	-1	0.480	0.472
28	-1	-1	1	-1	0.250	0.235
29	-1	-1	-1	1	0.480	0.463
30	0	0	0	0	0.581	0.581
31	1	-1	1	1	0.533	0.513

Table 3 Central composite design matrix of orthogonal values along with respective observed milk clotting observation

Run No.	Independent Variables				Milk Clotting Observation
	Orthogonal Values				
	A	B	C	D	
1	-1	1	-1	1	Slight precipitate along the sides of the tube, no curd formation after 30 min.
2	1	-1	-1	1	Slight precipitate along the sides of the tube, no curd formation after 30 min
3	0	0	0	-2	Granules appear on the sides of the test tube immediately, precipitated solution was formed after 20 min but not settled after 1 hr
4	1	-1	1	-1	Immediate milk clotting on the upper layer and complete precipitated solution after 30 min
5	0	0	0	2	Immediate slight granules formed, curd formation after 15 min.
6	-1	-1	-1	-1	No proper reaction after the addition of the enzyme
7	0	-2	0	0	Slight granules appeared on the upper layer, precipitated solution formed and not settled after 1 hr
8	-1	-1	1	1	Immediate milk clotting and no curd formation after 30min
9	2	0	0	0	Immediate Slight precipitate appears along the sides of the test tube, curd formation after 15 min and not settled after 1 hr
10	0	0	0	0	Immediate Slight precipitate appears along the sides of the test tube, curd formation after 15 min and settled after 1 hr
11	0	0	0	0	Immediate Slight precipitate appears along the sides of the test tube, curd formation after 15 min and not settled after 1 hr
12	1	1	-1	-1	Slight granules appears along the sides of the tube, no fine curd formation after 40 min
13	0	0	0	0	Good coagulation appears along the sides of the test tube and precipitated curd formation after 20min
14	0	2	0	0	Immediate Slight precipitate on the upper layer and curd formation at 15 min not settled after 1 hr.
15	1	1	-1	1	Slight granules appears along the sides of the tube, no fine curd formation after 40 min
16	0	0	0	0	Good coagulation appears along the sides of the test tube and precipitated curd formation after 20min
17	-1	1	1	-1	Immediate precipitate along the sides of the test tube and no complete milk clotting after 30 min
18	1	-1	-1	-1	Slight granules appears along the sides of the tube, no fine curd formation after 40 min
19	-1	1	1	1	Immediate precipitate along the sides of the test tube but no complete milk clotting
20	0	0	0	0	Immediate Good precipitate appears along the sides of the test tube and precipitated curd formation after 17 min
21	1	1	1	-1	Immediate slight granules appears on the upper layer of the tube, no curd formation after 30 min
22	0	0	-2	0	Immediate precipitate along the sides of the tube, complete precipitate and fine curd formation, settled after 25 min
23	1	1	1	1	Slight precipitate on the upper layer and precipitated solution formed after 30min, no fine curd formation after 1 hr
24	-2	0	0	0	Immediate Slight precipitate along the sides of the tube and precipitated solution formed after 1 hr and not settled after 1 hr
25	0	0	2	0	Immediate precipitate along the sides of the tube, complete precipitate and settled after 20 min.
26	0	0	0	0	Immediate Good coagulation appears along the sides of the test tube, curd formation after 15min
27	-1	1	-1	-1	Slight granules appears along the sides of the tube, no fine curd formation after 40 min
28	-1	-1	1	-1	Slight precipitate on the upper layer after the addition of the enzyme but no complete milk clotting after 30 min
29	-1	-1	-1	1	Slight granules appears along the sides of the tube, no fine curd formation after 40 min
30	0	0	0	0	Immediate Good coagulation appears along the sides of the test tube and precipitated curd formation after 15min
31	1	-1	1	1	Slight precipitate along the test tube after the addition of the enzyme and no fine curd formation after 30min

Table 4 Significance of regression coefficients for the production of milk clotting enzyme using Minitab 16 software

Model Term	Parameter estimate (Coefficients)		
Constant	0.580	31.901	0.000
A	0.032	3.271	0.005
B	0.017	1.788	0.093
C	-0.020	-2.110	0.051
D	0.024	2.534	0.022
A * A	-0.035	-3.967	0.001
B * B	-0.018	-2.080	0.054
C * C	-0.067	-7.449	0.000
D * D	-0.019	-2.205	0.042
A * B	-0.003	-0.311	0.760
A * C	0.010	0.882	0.391
A * D	-0.011	-0.924	0.369
B * C	0.016	1.349	0.196
B * D	-0.030	-2.532	0.022
C * D	0.036	3.062	0.007

- A, B, C, D = Linear effects
 A², B², C², D² = Squared effects
 AB, AC, AD, BC, BD, CD = Interaction effects
 A = Significant
 B = Significant
 C = Significant
 D = Significant
 A * A = Significant
 B * B = Significant
 C * C = Significant
 D * D = Significant

Table 5 Analysis of Variance (ANOVA) for the selected quadratic model for the Production of milk clotting enzyme

Sources of variation	Sum of squares	Degrees of Freedom	Mean square	F	P
Regression	0.259	14	0.018	7.99	0.000
Linear	0.057	4	0.014	6.19	0.003
Square	0.157	4	0.039	16.94	0.000
Interaction	0.044	6	0.007	3.22	0.029
Residual error	0.037	16	0.002		
Total	0.296	30			

- Linear = Significant
 Square = Significant
 Interaction = Significant

The student t distribution and corresponding p values, along with the parameter estimate were given in Table 4. The linear effect of all the parameters were found to be significant. The squared effects of all the parameters A², B², C², D² were also found to be significant and the coefficient of the effect of temperature (p = 0.0001) was found to be highly significant. The statistical significance of each term in the quadratic model was validated by the statistical tests called the Analysis-of-variance (ANOVA) and the results were given in Table 5. ANOVA of the regression model was significant and it was evident from the calculated F value (7.99) and a very low probability. The coefficient for the squared effect was highly significant (p=0.0001) when compared with the linear and interactive effects.

Response surface contour plots describe the relationship between the response and experimental levels of each variable and These plots explain the type of interaction between test variables and help to obtain the optimum conditions.. Fig3 to 5 shows the response surface plots against each of the independent variables while keeping the other variables at their '0' levels. The surface confined in the smallest curve of the response surface diagram indicated the maximum product yield. The elliptical nature of the contour indicates that this interaction is significant on the response. The response surfaces can also find the optimum range of process variables.

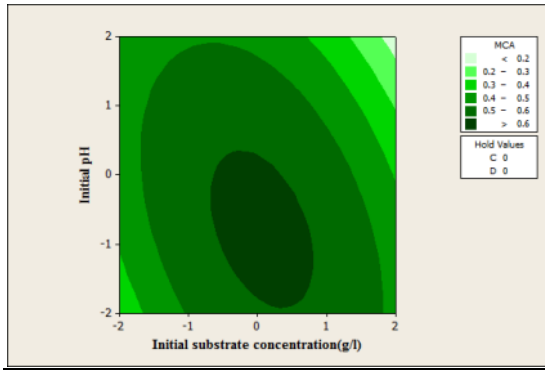


Fig.3 Response surface contour plot showing interactive effect of initial substrate concentration and initial pH on the production of milk clotting enzyme

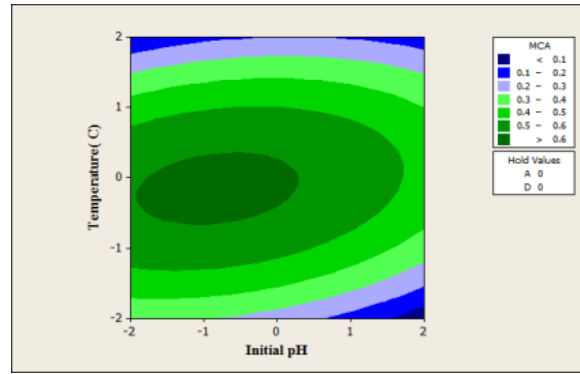


Fig.4 Response surface contour plot showing interactive effect of initial pH and temperature on the production of milk clotting enzyme

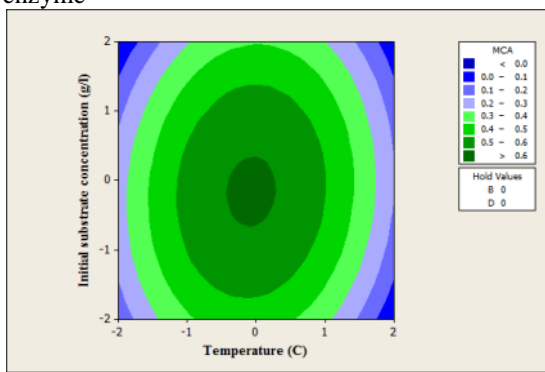


Fig.5 Response surface contour plot showing interactive effect of temperature and initial substrate concentration on the production of milk clotting enzyme

To validate the optimal parameters, confirmatory experiments were carried out by lab scale production. The observed results were compared with the predicted results. The process conditions for the maximum production of milk clotting enzyme by *Penicillium camemberti* under optimized conditions were given in Table 6. Milk Clotting Activity 0.585units/mg(MCA), Proteolytic Activity0.395units/mg (PA), the ratio MCA/PA1.44 and protein content 0.356mg/ml were found under optimum conditions . These values agree with the values from the response surface analysis (MCA=0.5933units/mg) confirming that the RSM using statistical design is the effective tool can be used to optimize the process parameters and to study the importance of individual, cumulative and interactive effects of the test variables in milk clotting enzyme production. The confirmatory experiments showed the high milk clotting activity and low proteolytic activity which is essential for the perfect milk coagulation. It was reported that the production of cheese is necessary to use rennin with strong milk clotting activity and the least proteolytic action to minimise dissolution of the curd. (Hashem.,1999)

Table 6 Optimum values of variables obtained from regression equations for the production of milk clotting enzyme by *Penicillium camemberti*

Parameter	Optimum value for milk clotting enzyme production
Initial Substrate Concentration (whey medium)(%v/v)	33
Initial pH	6.0
Temperature(°C)	40
Biomass Concentration (g/l)	10.3
Milk Clotting Activity (units/mg)	0.5933

IV. Conclusion

The production of milk clotting enzyme by *Penicillium camemberti* has been studied in submerged fermentation using whey as a substrate .The whey basal medium with casein shows the high milk clotting activity and it is an effective substrate for the production of milk clotting enzyme by *Penicillium camemberti*.

The results reported that the whey basal medium containing casein under static conditions enhanced the milk clotting activity of 0.585 units/mg with low proteolytic activity 0.404 units/mg. Statistical experimental design is an effective tool for studying the influence of process parameters on milk clotting activity. The results recommended that the whey medium is the high nutrient substrate for the production of milk clotting enzyme by the fungal culture *Penicillium camemberti*. It could be concluded that the whey could be used as a cheap source for the production of milk clotting enzyme as a medium for microbial growth.

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